Effect of a Synthetic Drilling Fluid (IPAR) on Antioxidant Enzymes and Peroxisome Proliferation in the American Lobster, *Homarsus americanus*

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EFFECT OF A SYNTHETIC DRILLING FLUID (IPAR) ON ANTIOXIDANT ENZYMES AND PEROXISOME PROLIFERATION IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*

by

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ABSTRACT

Hamoutene, D., Payne, J.F., Andrews, C.,Wells, J. and Guiney, J. 2004. Effect of a synthetic drilling fluid (IPAR) on antioxidant enzymes and peroxisome proliferation in the American lobster, *Homarus americanus*. Can. Tech. Rep. Fish. Aquat. Sci. 2554: iii +12 p.

The fluid component of drilling muds generally consists of water (water based mud: WBM), oil (oil based mud: OBM) or alternative synthetic fluids (synthetic-based mud: SBM) which are increasingly being used today. This study presents data on acute toxicity of IPAR , an isoalkane based synthetic fluid currently used in the Newfoundland offshore. Lobsters were dosed *per os* (1 mL every 3 days, 5 ml in total) and different aspects of lipid and protein metabolism were monitored. We investigated peroxisome proliferation as well as other endpoints including activities of antioxidant enzymes, protein levels and serum aminotransferases. The isoalkane mixture had no impact on peroxisome proliferation despite the high doses administered to animals. We found increased amounts of protein in claw muscles but these results were not accompanied by similar trends in other organs suggesting no adverse effect of IPAR. Serum aminotransferases showed a slight but non-significant increase. Given the very high levels of synthetic fluid to which the animals were exposed, results support the hypothesis that IPAR has little or no potential for adversely affecting the health of lobsters.

RÉSUMÉ

Hamoutene, D., Payne, J.F., Andrews, C.,Wells, J. and Guinney, J. 2004. Effect of a synthetic drilling fluid (IPAR) on antioxidant enzymes and peroxisome proliferation in the American lobster, *Homarus americanus*. Can. Tech. Rep. Fish. Aquat. Sci. 2554: iii + 12 p.

Les boues de forage contiennent une fraction liquide généralement constituée soit d'eau, soit d'hydrocarbures, soit d'autres produits synthétiques (esters, isoalcanes). Cette étude apporte des données sur la toxicité aigue d'un liquide de forage synthétique à base d'isoalcanes : IPAR. Après injection dans l'orifice buccal de homards (1 mL dans tous les 3 jours, 5 mL en totalité), certains aspects du métabolisme des animaux ont été étudiés. Nous avons examiné l'impact de IPAR sur les peroxisomes, des organites impliqués dans le métabolisme des lipides. De même, nous avons considéré l'activité de certaines enzymes antioxydantes, les taux de protéines solubles et les niveaux d'activités d'aminotransferases impliquées dans le métabolisme proteique. De façon globale, IPAR semble n'avoir aucun effet toxique sur le métabolisme du homard. Aucun impact sur la prolifération des peroxisomes n'a été observé. De plus, et malgré l'augmentation significative des protéines solubles des muscles, aucune augmentation protéique n'a été enregistrée pour les autres organes considérés (foie, branchies, cœur) suggérant l'absence d'impact majeur sur le métabolisme protéique. Une augmentation non significative des aminotransferases sériques est observée mais n'est confirmée par aucun autre signe de catabolisme accru. Malgré les doses importantes utilisées pour cette étude, IPAR ne semble provoquer aucune réponse sublethale chez les homards. Nos données confirment les résultats présentés par d'autres auteurs concernant la faible toxicité des boues à base synthétique.

INTRODUCTION

Drilling mud formulations are highly variable, containing any combination of over a thousand minor components (GESAMP 1993). The fluid component of drilling muds generally consists of water (water based mud: WBM), oil (oil based mud: OBM) or alternative synthetic fluids (synthetic-based mud: SBM) which are increasingly in use today. Some of these "low toxicity" fluids are mainly paraffinic oils and are free of polycyclic aromatic hydrocarbons. Most of the environmental toxicity data available concerns water or oil base muds; little information is available on the acute and chronic impact of synthetic fluids on marine organisms. An aliphatic hydrocarbon based drilling fluid, IPAR, is currently being used in the Newfoundland offshore. Payne et al. (1995) reported little effect on a variety of indices in flounder chronically exposed for 4 months to oil well cuttings enriched in relatively high levels of aliphatic hydrocarbons. Moreover, toxicity studies carried out on scallops, plankton, fish larvae and winter flounder indicated a low potential for acute toxicity (Armsworthy et al. 2000; Cranford et al. 2000; Payne et al. 2001). A more fundamental biochemical toxicological study showed that IPAR, could cause minor metabolic dysfunctions in rats (Wang 2000).Additional studies using Microtox and amphipod bioassays on authentic drill cuttings from the Newfoundland offshore also confirmed a low toxicity potential (Payne et al. 2001).

This study addresses the acute toxicity of a synthetic paraffinic drilling fluid: (IPAR) on a benthic crustacean: the American lobster. The bulk of wastes discharged from drilling platforms sediment rapidly and the accepted view is that any impact of particulate drilling wastes will be most severe on benthic animals (National Research Council 1983). In this study, we investigated the effect of IPAR on peroxisome proliferation as well as various serum and organ enzymes. Peroxisomes are ubiquitous single membrane-bound cell organelles containing a variety of hydrogen peroxide (H_2O_2)-producing oxidases and catalase (De Duve 1965). Peroxisomes are involved in different aspects of lipid metabolism and present the interesting characteristic of proliferating after treatment of animals with certain drugs and xenobiotics (Fahimi et al. 1982; Orbea et al. 1999). Peroxisome proliferation is characterized by an increase in peroxisome number and fractional volume, which is usually accompanied by the induction of catalase and/or palmitoyl co-A oxidase. Furthermore, peroxisome proliferation is associated with a decrease in serum cholesterol and/or triglycerides (Moody et al. 1983). In our study, we measured catalase activities in different organs of lobsters as well as serum triglycerides. We also evaluated peroxisome numbers by electron microscopy. Due to its lipidic nature (mainly alkanes), IPAR could be a potential inducer of peroxisome proliferation. Further endpoints considered in the present study included monitoring the activity of an antioxidant enzyme, superoxide dismutase (SOD), to detect any oxidative stress as well as serum levels of aminotransferase activities. Aspartate (AST) and alanine (ALT) aminotransferases can be taken as a measure of compensatory mechanisms as a consequence to impaired carbohydrate metabolism due to a chemical stress (Reddy et al. 1989). Moreover, AST and ALT activities can increase in serum when cellular damage is extensive resulting in leakage of the enzymes into the hemolymph system (Hoff et al. 2003).

MATERIAL AND METHODS

ANIMALS AND EXPERIMENTAL PROCEDURE

Two sets of 14 lobsters (≈0.7 kg) were used for this study. Upon acclimation in the aquariums, the first set (October 2003) was divided into 2 groups of 7 lobsters each and animals were injected *per os* with 1 mL of seawater (controls) or undiluted IPAR samples (exposed) every 3-4 days. Five injections were given in total and animals were sacrificed 1 day after the last injection. A second set of 14 lobsters were purchased one month later and the experiment was repeated under the exact same conditions. Animals were not fed during the duration of these experiments. Before sacrificing animals hemolymph was withdrawn and serum prepared by centrifugation at 2000 x g for 5 minutes. Samples were frozen before analysis. Pieces of 1 g of hepatopancreas, gill, heart and claw muscle were taken from every animal and S9 (10000 x g supernatant) samples were prepared immediately after homogenization in 4 mL of ice cooled Tris buffer (0.05M ph:7.4). Supernatant samples were frozen at -60 °C before analysis. A 1 mm^3 size piece of hepatopancreas was also sampled for analysis by electron microscopy. Electron microscopic observations were performed only on the first batch of lobsters.

PEROXISOME PREPARATION

Homogenates of hepatopancreas of the first batch of lobster were prepared in a Tris buffer (0.05 M, pH 7.4) and centrifuged for 15 minutes at 600 x g. The resulting supernatant was then centrifuged for 15 minutes at 15000 x g and the resulting pellets kept for analysis. The pellets represent a fraction enriched in peroxisomes (Biegel et al. 1995).

ENZYMATIC ANALYSIS

Superoxide dismutase

Enzymatic measurements were performed on S9 samples (hepatopancreas, gills, heart, claw muscle) according to Marklund and Marklund (1974). This method is based on the autoxidation of pyrogallol and activities are expressed as units of SOD/mg protein. Enzyme activities were assayed at room temperature. Samples and buffers were kept on ice prior to analysis.

Catalase

Measurement of H_2O_2 decomposition at 240 nm: The activity of catalase was quantified as previously described (Aebi, 1985). Briefly, catalase activity was measured by following the initial rate of decomposition of 30 mM H_2O_2 at 240 nm. The reaction mixture contained phosphate buffer and 100 µL of S9 supernatant; no detergent was used. Catalase activities were low and results were poorly reproducible in both hepatopancreas and gill samples. Thus, we used another technique to

evaluate catalase activity in hepatopancreas and "peroxisome" samples; no measurement were carried out on gill samples.

Oxygen release: This method is used to examine H_2O_2 decomposition by quantifying the oxygen produced after H_2O_2 is added to the sample assuming all conversion of peroxide to oxygen is due to catalase (Pamatmat 1988). Purified beef liver catalase was used as a standard and activities were expressed as units of catalase/ mg protein. Measurements were performed on hepatopancreas S9 samples as well as on "peroxisomes" prepared from lobster hepatopancreas.

ELECTRON MICROSCOPY

Analysis were carried out on electron micrographs of tissues processed for catalase cytochemistry according to (Orbea et al. 2002). Pieces of hepatopancreas (also called digestive gland) were sampled from the posterior extremity of the organ to insure homogeneity between electron microscopy sections when comparing animals. Small pieces of hepatopancreas were fixed by immersion for 1h at room temperature in 0.1 M cacodylate buffer pH 7.2 containing 2.5% NaCl, 6% sucrose and 2.5% glutaraldehyde. Tissues were then washed in the same buffer and kept overnight at 4°C. For catalase detection, hepatopancreas were preincubated in 0.01 M Teorell-Stenhagen buffer, pH 10.4, containing 5 mM DAB for 30 min at 37°C in darkness. Incubation was performed in the same medium plus 0.15% H_2O_2 for 2h. After washing in Teorell-Stenhagen buffer tissues were stored overnight prior to postfixation in reduced osmium for 1h at 4 °C. After washing in distilled water, tissues were dehydrated in different ethanol solutions and embedded in Epon. Semithin sections stained with Methylene Blue (0.5%) were examined by light microscopy for selection of appropriate regions for thin sectioning. Ultrathin sections counterstained with uranyl and lead acetate were observed under a ZEISS EM109 electron microscope. After identification of peroxisomes, counts were performed on 10-15 randomly selected fields of 0.0243 mm^2 for every section at a magnification of 10000. Electron microscopy studies were carried out at the microscopy laboratory of Memorial University, Newfoundland.

SERUM ANALYSIS

Serum analysis were carried out on a Beckman LX automated analyzer at the hematology/biochemistry laboratory of the General Hospital, St John's. Soluble protein levels were measured in S9 samples according to Lowry et al. (1951). Unpaired t-tests were performed to compare between results , $P \leq 0.05$ was considered significant.

RESULTS

Enzymatic analysis

No significant differences in SOD or catalase activities were found between S9 samples of control and exposed lobsters from either batch. A large difference in SOD levels was found between the 2 batches of lobsters (October-December 2003); the first group of lobsters having activity levels 4 to 10 times higher than the second one. The reason for these different levels of activities remains unclear. No seasonal factor is involved as lobsters were sampled one month apart. The only difference noted between the 2 batches of lobsters was the higher mortality in the first batch during the acclimation period (Table 1).

Catalase activity measured spectrophotometrically showed a low level of reproducibility for both hepatopancreas and gill samples. Thus, catalase was reassayed by measuring the release of O_2. No differences were found in oxygen production between control and exposed animals in hepatopancreas S9 samples (cont: 2681.8 ±1495.7; exp: 1981.6 ± 1783.7 units /mg prot. or peroxisomes (cont: 2396.6 ± 1058.9; exp: 1910.3 ± 1214.6 units/mg prot.).

Table 1. SOD and catalase activities in S9 lobster samples (results are expressed as means ± standard deviations).

		Controls (n=7 for every batch)				IPAR 5 injections (n=7 for every batch)			
Enzyme	Batch	Hp	Ht	G	CM	Hp	Ht	G	CM
SOD (units/mg prot.)	First batch	1234.60 ± 630.50	756.40 ± 483.90	296.40 ± 144.40	1157.20 ± 869.20	867.33 ± 503.50	479.20 ± 489.70	636.10 ± 393.20	835.00 ± 584.90
	Second batch	162.98 ± 74.56	137.63 ± 70.04	213.39 ± 222.81	108.58 ± 43.72	190.44 ± 131.75	124.21 ± 83.06	78.69 ± 71.64	96.58 ± 91.53
Catalase (nmoles H_2O_2/min/ mg prot.)	First batch	PD + NR	4.00 ± 3.92	PD + NR	2.79 ± 1.33	PD + NR	2.56 ± 1.72	PD + NR	1.14 ± 1.44
	Second batch	PD + NR	2.54 ± 1.88	PD + NR	1.94 ± 0.78	PD + NR	2.09 ± 1.43	PD + NR	2.50 ± 1.70

PD + NR: poor detection + no reproducibility
Hp: hepatopancreas, Ht: heart, G: gills, CM: claw muscle

ELECTRON MICROSCOPY

The tubules of the digestive gland of lobsters (we sampled the organ posterior extremities) have a rather simple construction and include a lumen, a digestive epithelium, basement membrane, contractile cells and tunica propria (Factor 1995). The peroxisome counts were performed mainly in the digestive epithelia with no cell type distinction of cell type (the digestive epithelium has been shown to comprise 4 cell types). Results expressed in number of peroxisomes/0.0243 mm^2 sections showed no differences between controls (17.30 ± 6.52 peroxisomes/0.0243 mm^2) and exposed (21.30 ± 7.65 peroxisomes/0.0243 mm^2). Peroxisomes had heterogeneous shapes and sizes and were concentrated in the vicinity of mitochondria and lipid droplets (Fig. 1 and 2).

SERUM ANALYSIS AND PROTEIN LEVELS IN S9

Soluble protein levels were measured in S9 prepared from hepatopancreas, gills, heart and claw muscles of lobsters. Measurements were performed on lobster samples from both batches as well as from previous groups of lobsters used for preliminary testing. A significant increase in soluble protein levels was found in S9 samples of claw muscle but not in other tissues after IPAR treatment (Table 2).

Serum levels of aminotransferase activities or triglycerides showed no significant difference between control and exposed lobsters (Table 3).

Table 2. Soluble protein levels in S9 samples (results are expressed as means ± standard deviations).

Organ	Controls (n=16)	IPAR exposed (n=17)
Hepatopancreas (mg/ml eq. mg/4 g wet weight)	16.10 ± 3.63	14.42 ± 3.03
Gills (mg/ml)	5.84 ± 1.58	6.38 ± 1.68
Heart (mg/ml)	10.03 ± 3.62	10.09 ± 2.20
Claw muscle (mg/ml)	6.74 ± 1.92	8.82 ± 2.83 *

*significantly different from controls, t-test, p=0.02

Table 3. AST and ALT levels in lobster serum (results are expressed as means ± standard deviations).

Blood test	Batch	Controls (n=5 for every batch)	IPAR (n=5 for every batch)
AST (units/L)	First batch	21.25 ± 6.65	31.75 ± 22.20
	Second batch	15.00 ± 6.96	20.00 ± 6.67
ALT (units/L)	First batch	13.50 ± 2.38	20.50 ± 10.08
	Second batch	10.17 ± 7.11	10.80 ± 9.68
Triglycerides (mmol/L)	First batch	0.12 ± 0.03	0.18 ± 0.13
	Second batch	0.06 ± 0.03	0.10 ± 0.06

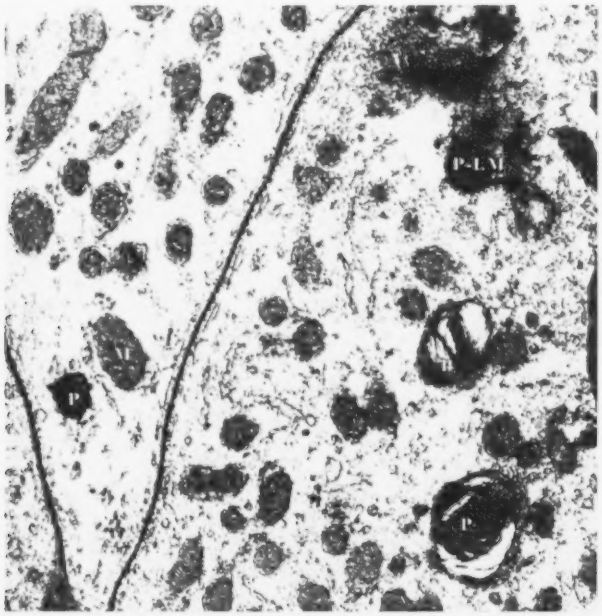

Figure 1. Electron micrograph from the hepatopancreas of a control lobster showing overall distribution and DAB reaction of peroxisomes (P) (x 24,500). P-LM: peroxisome without a distinct limiting membrane, M: mitochondria.

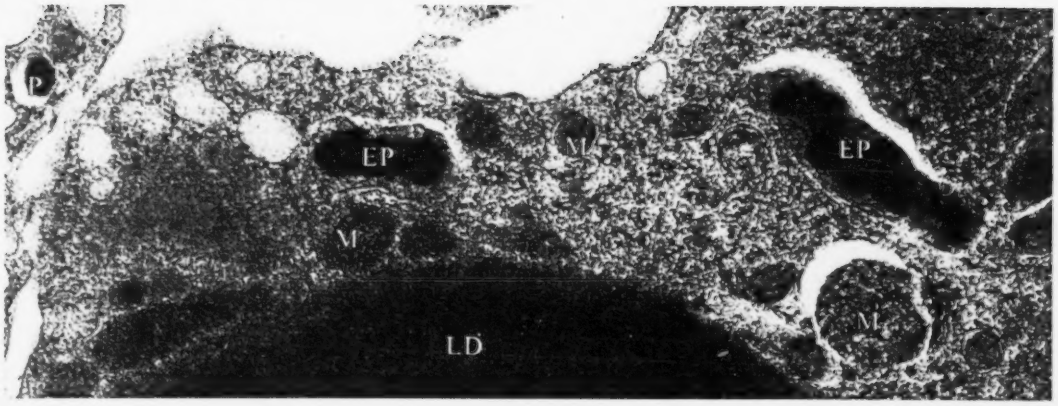

Figure 2. Electron micrograph from the hepatopancreas of a lobster injected with IPAR showing the distribution, overall distribution and DAB reaction of peroxisomes (x 24,500). EP: elongated peroxisome, M: mitochondria, LD: lipid droplet. No differences in peroxisome numbers or structure (similar elongated peroxisomes were observed in control) were observed between controls and IPAR exposed lobsters.

DISCUSSION

There is paucity of data on acute toxicity of drilling fluids of any kind, likely because of their low acute toxicity (Holdway 2002). The environmental impact of SBMs which are proposed to replace OBMs owing to their technical and environmental advantages (Burke and Veil 1995) is even less known. This study addresses the acute sublethal toxicity of IPAR; an ultra-pure isoalkane with no aromatics and sulfur compounds (www.expochem.com). Experiments were conducted by injecting lobsters *per os* with IPAR samples. This is a more realistic pathway than injection directly into the body cavity. It is also important to note that the animals were exposed to very high levels of the fluid, which would not be expected under field conditions.

In mammals, peroxisomes play a central role in many diverse aspects of lipid metabolism. As IPAR contains mainly alkanes it seemed necessary to investigate any acute effect on peroxisome proliferation. It is important to correlate peroxisome numbers determined by electron microscopy with putative biomarkers of peroxisome proliferation (Ringeissen et al. 2003). In this study, we investigated catalase activities as well as serum triglyceride content. Our results show no effect of IPAR on catalase activity, triglycerides or peroxisome numbers suggesting that IPAR base muds are not likely to cause peroxisomal proliferation in lobsters. Preliminary attempts to measure palmitoyl co-A oxidase, another marker of peroxisome proliferation, in lobster peroxisomes were unsuccessful for unknown reasons (results not shown). Wang (2000) found an increase in

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palmitoyl co-A oxidase activities after 4 injections of IPAR in rats but no effect was observed after one or two doses of the fluid. The study of peroxisomes in invertebrates deserves greater attention, especially because existing data suggests that these organelles can be used as toxicity markers (Orbea et al. 1999, 2002). Studies on peroxisomes in the hepatopancreas of decapod are generally lacking (Lobo Da Cunha 1995). Our study shows organelles of different shapes and sizes (0.2-0.82 μm in diameter) located around mitochondria and lipid droplets. Organelle size was close to those of peroxisomes described in crab liver samples (Lobo Da Cunha 1995). Moreover, similar elongated peroxisome profiles were observed (Fig. 2). Figure 1 reveals some peroxisomes which lacked a distinct limiting membrane, an observation which has been previously reported by authors who concluded that storage of glutaraldehyde-fixed sections of rat liver in various buffers and aqueous media results in progressive deterioration of peroxisome structure (Fahimi 1974). This applies to our study as hepatopancreas samples were kept at 4 degrees (for practical reasons) 12h before DAB treatment and 12h prior to postfixation and embedding.

Metabolism of xenobiotics may result in the formation of reactive oxygen species (ROS). Thus, aquatic organisms are usually subject to an enhanced oxidative stress due to acute or chronic exposure to pollutants in their environments. The primary defence against oxidative damage consists of some low molecular weight compounds (vitamins, glutathione) and the major antioxidant enzymes catalase, superoxide dismutase (SOD) and glutathione peroxidase (Winston 1991). Similarly to catalase we found no differences in SOD activity after acute exposure to IPAR showing no oxidative stress in all organs investigated. A significant difference was observed between the two batches of lobsters used in our experiments. Abele et al. (1998) have found seasonality in SOD and catalase activity in several invertebrates from North Sea mud flats. Nonetheless, this explanation does not apply to our study as lobsters were sampled one month apart. Angel et al. (1999) found significant differences in SOD activities of a benthic bivalve sampled in the same month during two subsequent years. There were no significant differences in seawater temperature, bivalve age structure or other obvious factors that could affect SOD activity when comparing the two years; the reason for such different results remain unclear (Angel et al. 1999). The only noticeable difference between the two batches of lobsters was an elevated mortality during the acclimation period for the first group suggesting a higher level of stress that could be associated with a higher SOD activity.

A significant increase in soluble proteins was found in claw muscle samples after IPAR exposure; no increase was observed in gills, hepatopancreas or heart. Sreenivasulu and Reddy (1997) found similar increases in soluble proteins in prawn tissues after exposure to an organophosphorus insecticide. The increase in tissue soluble proteins could reflect an increase in the synthesis of enzymes responsible for the detoxification of insecticides (Sreenivasulu and Reddy 1997). This is probably not applicable to our study, since the increased protein levels observed in muscle tissues of prawns were accompanied by higher values in hepatopancreas and gills; furthermore, the prawns were exposed to a compound of known toxicity. On the other hand, Reddy and Venugopal (1991) found an association between a decrease in protein contents in crab tissues and higher transaminase activities after exposure to very high levels of cadmium. Similarly, Radhakrishnaiah et al. (1995) observed a decrease in muscle proteins of a freshwater field crab

after exposure to both lethal and sublethal pesticide concentrations. A toxic stress could provoke a time-dependent depletion in amino acid content and enhanced activities in transaminases of crab tissues to satisfy higher energy needs (Reddy et al. 1989; Reddy and Venugopal 1991; Radhakrishnaiah et al. 1995). A non-significant elevation in serum transaminases was observed in both batches of lobsters after IPAR injection but no other indication of increased catabolism was observed. As previously described in fish (Neff 1985), and irrespective of changes in protein content, an increase in ALT and AST activity could also indicate membrane damage leading to the release of enzymes into the serum. Moreover, an increase in proteins could favor structural rigidity as a strategy of adaptation to an ambient toxic medium (Radhakrishnaiah et al. 1995). Nonetheless, this "fortification" process is mainly observed in gills to ensure a normal respiratory activity (Radhakrishnaiah et al. 1995) and is indicative of a toxic response that did not seem to occur in this study with lobster.

Overall, the results of this study with lobster supports earlier studies indicating that alkane based drilling fluids should have little or no ecotoxicological potential.

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